

Dual Stimulation of Epstein-Barr Virus (EBV)-Specific CD4⁺- and CD8⁺-T-Cell Responses by a Chimeric Antigen Construct: Potential Therapeutic Vaccine for EBV-Positive Nasopharyngeal Carcinoma

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Virus-associated malignancies are potential targets for immunotherapeutic vaccines aiming to stimulate T-cell responses against viral antigens expressed in tumor cells. Epstein-Barr virus (EBV)-associated nasopharyngeal carcinoma, a high-incidence tumor in southern China, expresses a limited set of EBV proteins, including the nuclear antigen EBNA1, an abundant source of HLA class II-restricted CD4⁺ T-cell epitopes, and the latent membrane protein LMP2, a source of subdominant CD8⁺ T-cell epitopes presented by HLA class I alleles common in the Chinese population. We used appropriately modified gene sequences from a Chinese EBV strain to generate a modified vaccinia virus Ankara recombinant, MVA-EL, expressing the CD4 epitope-rich C-terminal domain of EBNA1 fused to full-length LMP2. The endogenously expressed fusion protein EL is efficiently processed via the HLA class I pathway, and MVA-EL-infected dendritic cells selectively reactivate LMP2-specific CD8⁺ memory T-cell responses from immune donors in vitro. Surprisingly, endogenously expressed EL also directly accesses the HLA class II presentation pathway and, unlike endogenously expressed EBNA1 itself, efficiently reactivates CD4⁺ memory T-cell responses in vitro. This unscheduled access to the HLA class II pathway is coincident with EL-mediated redirection of the EBNA1 domain from its native nuclear location to dense cytoplasmic patches. Given its immunogenicity to both CD4⁺ and CD8⁺ T cells, MVA-EL has potential as a therapeutic vaccine in the context of nasopharyngeal carcinoma.

There is considerable interest in the immunotherapeutic targeting of virus-associated human tumors with T cells, particularly CD8⁺ cytotoxic T cells, specific for viral proteins expressed in the tumor. Epstein-Barr virus (EBV)-associated malignancies are prime examples of such tumors, and the successful use of adoptively transferred virus-specific T-cell preparations to treat EBV-positive posttransplant lymphomas (38, 39) constitutes an important proof of the immunotherapeutic principle. These lymphomas, especially the early posttransplant lesions, express the full spectrum of eight latent virus proteins and, as such, resemble the lymphoblastoid cell lines (LCLs) generated by EBV transformation of normal B cells in vitro (18, 32). Importantly, protein expression includes the immunodominant nuclear antigens EBNA 3A, 3B, and 3C, which are the principal targets recognized by EBV-specific effector T-cell preparations of the kind produced by LCL stimulation in vitro and used in current adoptive transfer protocols (37). The challenge now is to devise new strategies, based on either adoptive T-cell transfer or vaccination, that will target other EBV-associated tumors with more restricted patterns of latent protein expression that exclude the EBNA3 antigens.

One such tumor, nasopharyngeal carcinoma, is seen worldwide but is particularly common in southeast Asia, where in certain areas the incidence can reach 50 cases per 100,000

population per year (35). Viral antigen expression in nasopharyngeal carcinoma cells (18) is limited to the nuclear antigen EBNA1, a sequence-specific DNA binding protein involved in EBV episomal genome maintenance and gene transactivation (52), and the latent membrane proteins LMP1, a major effector of virus-induced cellular change (30) but only detectable in 30 to 50% of tumors, and LMP2, a protein with signaling properties dependent upon its interaction with cellular tyrosine kinases (25). Of these three viral proteins, EBNA1 cannot be exploited as a target for CD8⁺ T cells because the endogenously expressed EBNA1 protein is protected from proteasomal degradation by the presence of a large glycine-alanine repeat (GAR) domain (2, 23). Furthermore, LMP1 is poorly immunogenic for the CD8⁺ system, whereas LMP2 is a source of subdominant epitopes eliciting weak but nevertheless detectable responses restricted through several HLA class I alleles, including some, such as A11.01, A24.02, and B40.01, that are common in the Chinese population (21).

A first attempt at therapeutic vaccination of nasopharyngeal carcinoma patients with CD8 epitope peptides from LMP2 gave encouraging early results, but the epitope-specific responses seen were only maintained for a few months (24). This may reflect a need for accompanying CD4⁺ T-cell help because CD4⁺ T cells are increasingly thought to be important in maintaining an effective CD8⁺-T-cell response (16, 43, 46) and may also be able to act as effector cells in their own right (51). In that context EBNA1 is known to be immunodominant over LMP1 and LMP2 as a target for CD4⁺-T-cell responses as measured by gamma interferon (IFN- γ) release in an Elispot assay (22), and indeed some EBNA1-specific CD4⁺-T-cell

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clones appear capable of recognizing HLA class II-positive target cells endogenously expressing the EBNA1 protein (31, 34). Immunotherapeutic targeting of nasopharyngeal carcinoma, a tumor that expresses both HLA class I and class II antigens, would therefore seek to recruit both CD4⁺ and CD8⁺ immunity.

The present paper describes work towards that end, focusing on a vaccine-based approach rather than on adoptive T-cell transfer. As a vaccine vector we chose modified vaccinia virus Ankara (MVA), a highly attenuated derivative of vaccinia virus with an excellent safety record (27, 45). When used in recombinant form to express foreign antigens in animal systems, MVA-based vaccines have been effective not just prophylactically against viral (12, 14, 48, 53) and parasitic (33, 41) infections, but also therapeutically against antigen-expressing tumors (3). As a vaccine antigen capable of targeting responses against nasopharyngeal carcinoma, we constructed an EBNA1-LMP2 fusion protein which, for reasons of safety, has been manipulated to destroy the gene-transactivating and signaling abilities of its individual component proteins but retains multiple CD4⁺ and CD8⁺ epitopes in its primary sequence.

MATERIALS AND METHODS

Construction of the EBNA1-LMP2 gene fusion. The 3' half of the EBNA1 open reading frame (codons 363 to 641) was amplified from a reference type 1 Chinese EBV isolate from a nasopharyngeal carcinoma patient with the primers E1For (5'-AGTCGGTACCGCCACCATGGGGAGGAGTCGTGAAAGAG) and E1Rev (5'-CATCTCTGCCCTTCCTACC). The italicized (non-EBV) sequence within E1For includes an additional *KpnI* restriction enzyme site (underlined), a Kozak consensus sequence, and an ATG start codon. Nucleotides 4 to 21 of the E1Rev sequence are complementary to the final 18 nucleotides of the EBNA1 open reading frame, and nucleotides 1 to 3 of E1Rev are complementary to the first three nucleotides of LMP2.

The full-length LMP2 open reading frame was amplified from a previously cloned LMP2 cDNA (kindly provided by Y. S. Chang, Chang-Gung University, Taiwan), again of type 1 Chinese EBV strain origin, with primers L2For (5'-GAGGAAGGGCAGGAGATGGGGTCCCTAGAAATGG) and L2Rev (5'-GAA TGCATGCGGCGCTATACAGTGTGCGATATGG). The L2For sequence contains at its 5' end 15 nucleotides complementary to nucleotides 4 to 18 of the E1Rev primer. The italicized (non-EBV) sequence within L2Rev includes an additional *NotI* restriction enzyme site (underlined) overlapping the complementary sequence of the native TAG stop codon of LMP2 (bold).

The PCR amplicons from both separate first-round amplifications were purified, mixed together, and then reamplified with primers E1For and L2Rev, producing a fusion of EBNA1 and LMP2 (henceforth referred to as EL) without the introduction of any additional DNA sequence within the EBNA1-LMP2 coding region. The fusion was cloned into plasmid pUC18 via restriction enzyme sites for *KpnI* and *NotI*. Finally, two tyrosine codons within the LMP2 coding region of the EL fusion gene were mutated to phenylalanine codons by two successive rounds of inverse PCR with PFU Turbo polymerase (Stratagene). The first PCR used primers 74For (5'-TTTCAACCACTAGGAACCCAAG) and 74Rev (5'-GTCCGAGTGACGGTCGCCA) to mutate codon 74 (underlined). The second round of PCR used primers 112For (5'-TTTGAAGAAGCGGGCAGAGG) and 112Rev (5'-TATGTGTTGAGATGAGTCATCC) to mutate codon 112 (underlined).

Production of MVA recombinants. For expression from the MVA vector, the sequenced EL fusion gene was subcloned from the pUC18 vector into a modified version of the vaccinia virus shuttle vector pSC11 which contained *KpnI* and *NotI* restriction enzyme sites (kindly provided by Vincenzo Cerundolo, Institute of Molecular Medicine, University of Oxford). The MVA-EL recombinant was generated by homologous recombination in primary chicken embryo fibroblast (CEF) cells, first transfected with the EL shuttle vector and then infected with the parental MVA strain. MVA-EL was subsequently isolated by repeated rounds of plaque purification on CEF cells, with selection for β -galactosidase expression. Plaque-purified virus was amplified in CEF cells and stocks were titrated on BHK21 cells.

In parallel, we constructed an MVA recombinant, MVA-E1ΔGA, expressing

a GAR-deleted form of the EBNA1 protein and another recombinant, MVA-LAMP-E1ΔGA, expressing the above protein targeted to the lysosomal compartment by the addition of an endoplasmic reticulum signal sequence and a lysosome-associated membrane protein (LAMP) target sequence (11). Additional control viruses MVA-pSC11 (empty vector) and MVA-LMP2 were provided by Neil Blake (University of Liverpool).

Protein expression from MVA recombinants. Expression of protein from the recombinant viruses was first confirmed by infecting HeLa cells for 1 h at a multiplicity of infection of 10. The cells were then washed and cultured for a further 18 h before harvesting. Cellular protein extracts were prepared and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a Novex 4 to 12% Nupage bis-Tris gel (Invitrogen). Polypeptides were transferred to a nitrocellulose membrane and probed for expression with monoclonal antibodies (MAbs) 1H4-1 (10) and OT1x (4) for EBNA1 and MAb 14B7 (9) for LMP2. Blots were developed with enhanced chemiluminescence (Amersham).

For localization of MVA-expressed proteins, A431 cells growing on glass slides or matured dendritic cells (see later) in suspension were infected with MVA viruses for 1 h, then washed and incubated for a further 18 h. Cells were fixed with paraformaldehyde (in the case of dendritic cells after adherence to poly-L-lysine-coated glass coverslips), permeabilized with 0.1% Triton X-100, and stained with the appropriate rat MAbs 1H4-1 and 14B7 (9). Cells were costained with mouse MAbs to HLA-DR (L243, Immunotech), to early endosomal antigen EEA1 (TL-c14, Transduction Laboratories) and CD63 (6H1) (1) as endosomal markers, and to lysosome-associated membrane protein LAMP1 (TL-c25, Transduction Laboratories) as an endosome/lysosome marker. The labeled second step antibodies were anti-rat Alexa 488 and anti-mouse Alexa 594 (both from Molecular Probes).

Analysis of EBNA1/LMP2 function. For functional assays, the EL fusion gene was subcloned with *KpnI* and *NotI* restriction enzymes from the pSC11 shuttle vector into the eukaryotic expression plasmid pCDNA3. The plasmid pSG5-EBNA1 expressing wild-type EBNA1 and the pCDNA3 empty vector served as positive and negative controls, respectively, in the EBNA1 functional assays. Transfection efficiencies were normalized by including a control β -galactosidase-expressing plasmid in all transfections. To measure EBNA1 transactivation activity, HeLa cells were transfected with Fugene (Roche Molecular Biosciences) with 10 μ g of test plasmid, 1 μ g of the reporter gene plasmid FR-TK-Luc (29), and 1 μ g of the β -galactosidase transfection efficiency control; after 48 h luciferase activity was measured with luciferin (Promega) and normalized to β -galactosidase expression detected with Galacto-light substrate (Tropix) according to the manufacturer's recommended protocols.

To measure EBNA1 DNA binding activity, DG75 cells were transfected by electroporation with 10 μ g of test plasmid, 2 μ g of the qp-Luc reporter gene plasmid (40), and 2 μ g of the β -galactosidase transfection efficiency control; after 24 h luciferase and β -galactosidase were measured as described above.

Functional LMP2 activity was determined by phosphorylation of the Akt cellular protein as a readout (42, 49). HeLa cells were transfected with 1 μ g of pKB-HA, expressing a hemagglutinin (HA)-tagged version of the Akt molecule. Cells were also transfected with 1 μ g of test plasmid, pCDNA3-EL, or control plasmid pCDNA3 (empty vector), pSG5-LMP2, pSG5-LMP2B, or p110^{CAAX}. Cells were transfected for a total of 48 h and serum starved for the final 24 h of incubation. Cultures were lysed, and 200 μ g of cell lysate was immunoprecipitated with anti-HA antibody as described (7). Phosphorylated Akt was detected by SDS-PAGE and immunoblotting with an Akt phosphoserine 473-specific antibody (Cell Signaling Technology).

Blood donors, cell preparations, and cell lines. Blood samples were obtained from healthy EBV-seropositive donors of Caucasian or Chinese origin and of known HLA type. For in vitro reactivation experiments, peripheral blood mononuclear cells (PBMCs) were separated by standard Ficoll-Hypaque centrifugation into standard culture medium RPMI 1640 (Invitrogen) supplemented with 2 mM glutamine, 100 IU of penicillin per ml, 100 μ g of streptomycin per ml, and 5% autologous serum. Dendritic cells were prepared as described (22) by 6 days of culture of adherent PBMCs in granulocyte-macrophage colony-stimulating factor- and interleukin-4-supplemented medium, again with 5% autologous serum, and then matured for 24 h in 50 ng of tumor necrosis factor α /ml.

EBV (B95.8 strain)-transformed LCLs and EBV-negative B-cell blasts (CD40 ligand and interleukin-4 stimulated) were prepared by standard methods, and the transporter associated with antigen processing (TAP)-negative T2 and T2:B35 cell lines were as described (20). Primary chicken embryo fibroblast cells, produced from eggs obtained from a specific-pathogen-free colony (Institute for Animal Health, Compton, United Kingdom), and cell lines BHK-21, HeLa, and A431, obtained from the European Collection of Animal Cell Cultures, were maintained in Dulbecco's modified Eagle's medium supplemented with 2 mM

glutamine, 100 IU of penicillin per ml, 100 μ g of streptomycin per ml, and 10% fetal calf serum.

In vitro reactivation and cloning. Matured dendritic cell preparations were infected with MVA EL virus for 1 h at a multiplicity of infection of 2. The following day, autologous PBMCs were mixed with dendritic cells at a 40:1 ratio and cocultured in a 2-ml well with medium containing 5% autologous serum. As a control, PBMCs were stimulated in parallel with γ -irradiated (4,000 rads) autologous LCL cells at the same responder-stimulator cell ratio. Cells were restimulated on day 11 by addition of freshly prepared virus-infected dendritic cells or irradiated LCL as applicable.

In some experiments, cultures were harvested before restimulation on day 11 and immediately cloned by limiting dilution at 3 cells per well on γ -irradiated autologous LCL (10^4 cells/well) and γ -irradiated phytohemagglutinin (PHA) treated allogeneic PBMCs (10^5 cells/well) in interleukin-2-supplemented medium with fetal calf serum as described (21). Growing microcultures were transferred to 2-ml wells and further expanded by stimulation with γ -irradiated autologous LCL and γ -irradiated allogeneic PBMCs in interleukin-2-supplemented medium as before.

T-cell recognition assays. CD8⁺-T-cell recognition of MVA-expressed antigen was measured in standard 5-h chromium release assays with, as effector cells, epitope-specific CD8⁺-T-cell clones prepared by conventional LCL stimulation and selection as described (2, 21) and, as target cells, LCLs either infected overnight with MVA viruses at a multiplicity of infection of 10 or preexposed for 1 h to 5 μ M epitope peptide or an equivalent concentration of dimethyl sulfoxide solvent as a control. CD8⁺-T-cell clones were against the following epitopes (identified by the first three letters of the peptide sequence): HLA A2-restricted epitopes LLW (LMP2 amino acids 329 to 337) and FLY (LMP2 amino acids 356 to 364), the HLA A11-restricted epitope SSC (LMP2 amino acids 340 to 350), the HLA A24-restricted epitope TYG (LMP2 amino acids 419 to 427), and the HLA B35.01-restricted epitope HPV (EBNA1 amino acids 407 to 417).

CD4⁺-T-cell recognition of MVA-expressed protein was measured by IFN- γ enzyme-linked immunosorbent assay (Endogen) following the manufacturer's recommended protocol, with CD4⁺-T-cell clones prepared as described (22) or generated in the present work. Epitope peptides were synthesized with 9-fluorenylmethoxycarbonyl chemistry (Alta Bioscience, University of Birmingham), dissolved in dimethyl sulfoxide, and their concentrations were determined by biuret assay.

The induction both of CD4⁺ and CD8⁺ epitope-specific responses in *in vitro* reactivation experiments was detected by Elispot assay of IFN- γ -producing cells with irradiated (4,000 rads) autologous PBMCs or autologous B-cell blasts pulsed with 5 μ M peptide or dimethyl sulfoxide solvent as presenting cells. Different numbers of the polyclonal T-cell cultures were added and incubated overnight before development of the assay as described previously (22).

RESULTS

Cloning and expression of the EL fusion protein. With a PCR-based approach, we generated a gene fusion which contained the 3' half of the EBNA1 open reading frame (EBNA1 codons 363 to 641, lacking the N terminus and GAR domain codons but encoding most of the known CD4⁺-T-cell epitopes) immediately upstream of a full-length LMP2 cDNA (LMP2 codons 1 to 497). As shown in Fig. 1A, the fusion gene was further modified by inverse PCR mutagenesis to convert the codons for two key signaling residues in LMP2, tyrosine 74 and tyrosine 112, to phenylalanines (25). This EL gene fusion was then inserted into the parental MVA genome by homologous recombination to produce the recombinant virus MVA-EL. After plaque purification of this virus and sequencing of the EL gene to confirm its integrity in the recombinant virus genome, MVA-EL was tested for expression of the EL fusion protein.

Figure 1B shows immunoblots made from HeLa cells 18 h after infection at a multiplicity of infection of 10 with MVA-EL or one of the following control viruses: MVA-pSC11 (empty vector), MVA-E1 Δ GA, expressing a GAR-deleted form of the EBNA1 protein, and MVA-LMP2, expressing full-length LMP2. For the EBNA1 blot, an equivalent loading of protein

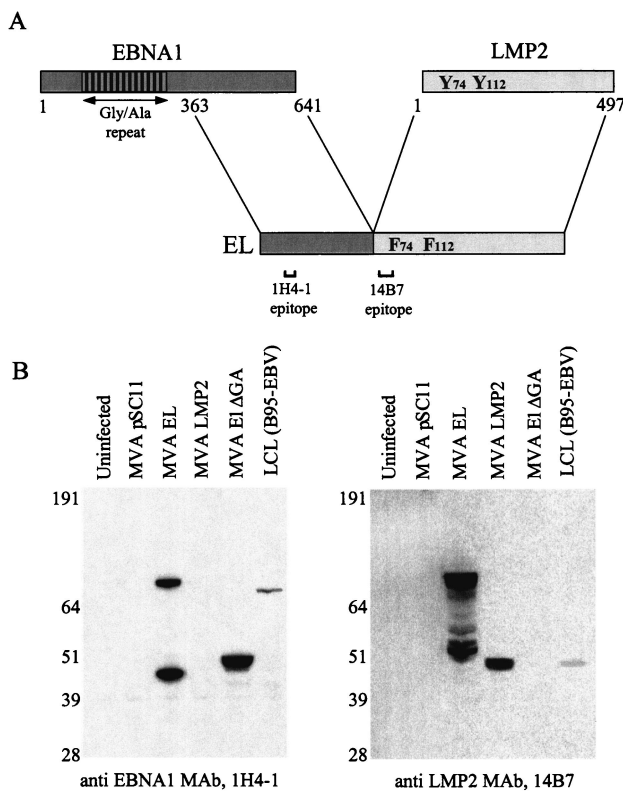


FIG. 1. (A) Schematic diagram showing the composition of the EL fusion construct and the locations of MAb-defined epitopes. (B) Detection of the EL fusion protein in MVA-EL-infected HeLa cells with the anti-EBNA1 MAb 1H4-1 and the anti-LMP2 MAb 14B7. Other tracks are loaded with protein from HeLa cells or HeLa cells infected with the MVA-pSC11 control virus, with MVA-LMP2, or with MVA-E1 Δ GA. Protein extracts from an EBV (B95.8 strain)-transformed LCL were used as an additional reference. Sizes are shown in kilodaltons.

from an EBV (B95.8 strain)-transformed LCL is shown in the right-hand lane for comparison, whereas for the LMP2 blot, 10-fold more LCL cell protein was loaded to allow detection of the naturally expressed antigen. In MVA-EL-infected cells, a protein running at \approx 90 kDa was detected both by the 1H4-1 MAb, specific for an epitope now localized to residues 439 to 442 in the EBNA1 C-terminal domain (F. Grassier, personal communication), and by the 14B7 MAb, specific for an epitope between residues 37 and 64 in the LMP2 N-terminal domain (9).

This EL fusion protein is clearly larger than wild-type EBNA1 (running at 85 kDa), GAR-deleted EBNA1 (running at 52 kDa) and wild-type LMP2 (running at 54 kDa). Note also that the two MAbs each detected different smaller species in the MVA-EL track. Since PCR analysis of the MVA-EL virus stock never gave evidence of a truncated fusion gene (data not shown), these smaller species appear to be breakdown products of the EL fusion protein. There appears to be one major breakdown product containing the 1H4-1 MAb epitope whose size is consistent with a fragment containing most of the EBNA1 sequences from EL, while the 14B7 MAb detected a series of truncated proteins all larger than wild-type LMP2 but either lacking the 1H4-1 epitope or with the epitope occluded.

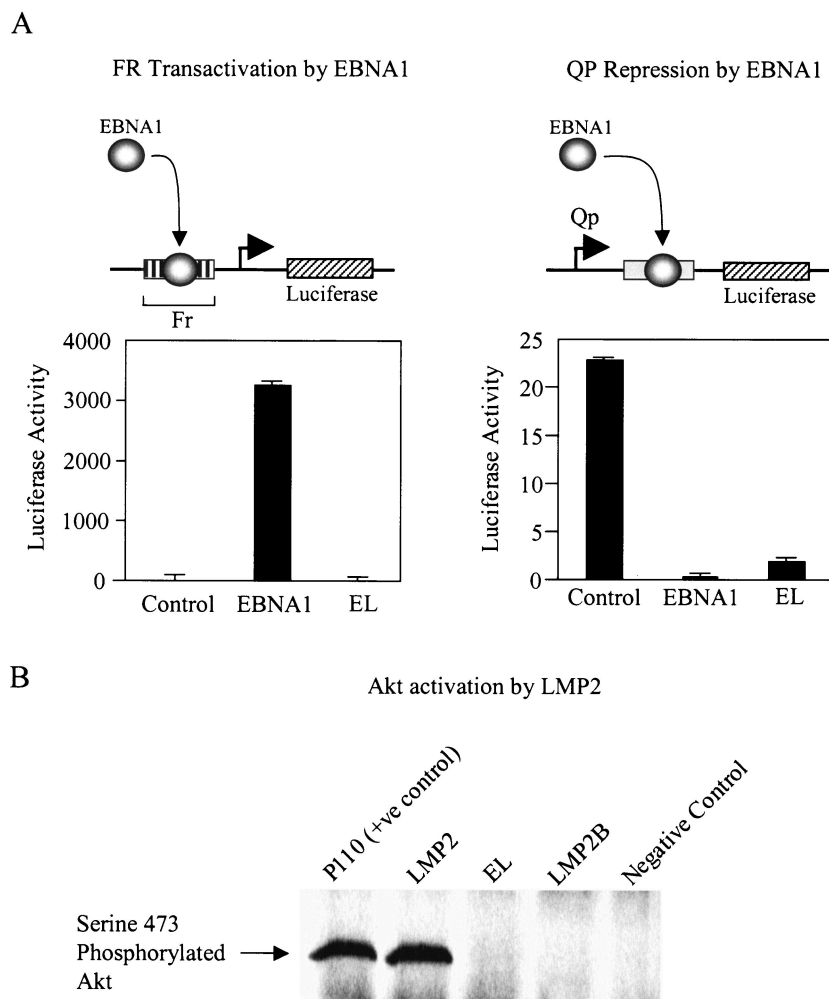


FIG. 2. Functional analysis of the EL fusion protein. (A) Transient transfection assays with the FR-TK-Luc reporter in HeLa cells and the Qp-Luc reporter in DG75 cells. Cells were cotransfected with the relevant reporter and with either a control plasmid or plasmids expressing full-length EBNA1 or the EL fusion protein. Results are expressed as luciferase activity in arbitrary units. (B) Transient transfection assay measuring phosphorylation of HA-tagged Akt. An HA-tagged Akt expression plasmid was cotransfected with an expression plasmid for full-length LMP2, for EL, for LMP2B, or (as a positive control) for p110. The HA-tagged Akt was immunoprecipitated with an HA-specific antibody, separated by SDS-PAGE, and blotted with a phosphoserine 473 Akt-specific antibody.

Functional inactivity of the EL fusion protein. We next performed a number of assays to see whether the EL fusion protein, in this case expressed by transient transfection from a plasmid vector, had retained the transactivating ability of wild-type EBNA1 or the signaling ability of wild-type LMP2. Figure 2A (left) shows the results of a cotransfection assay in HeLa cells with a reporter construct carrying multiple copies of an EBNA1 binding site (the family of repeats [FR] native to the EBV origin of plasmid replication, *oriP*) upstream of a luciferase gene (19). Cotransfection of this reporter with a wild-type EBNA1-expressing plasmid led to strong luciferase expression, whereas cotransfection with the EL-expressing plasmid gave no transactivation. Interestingly, however, a second type of cotransfection assay suggested that the EL fusion protein retained the capacity to bind DNA specifically. This assay utilizes a luciferase reporter downstream of the *Bam*HI-Q promoter from the EBV genome, a promoter which wild-type EBNA1 normally represses by binding to the adja-

cent low-affinity consensus sites downstream of the transcription start (40). As shown in Fig. 2A (right), EL was almost as efficient as wild-type EBNA1 in repressing Qp-driven luciferase expression.

A further set of cotransfection assays were carried out to study a downstream marker of LMP2-mediated signaling, namely a phosphatidylinositol 3 kinase-dependent phosphorylation of the cellular Akt protein (42, 49). HeLa cells were transfected with plasmids expressing an HA-tagged form of Akt and either EL, wild-type LMP2, the LMP2B protein (a naturally occurring isoform of LMP2 lacking the N-terminal signaling domain residues 1 to 119), or the phosphatidylinositol 3 kinase subunit P110 that normally targets Akt (5). Figure 2B shows the results obtained when HA-tagged Akt was immunoprecipitated from cotransfected cells, immunoblotted, and probed with an antibody specific for the phosphorylated serine residue 473 within Akt. Both the positive control p110 and wild-type LMP2 were capable of inducing strong Akt phos-

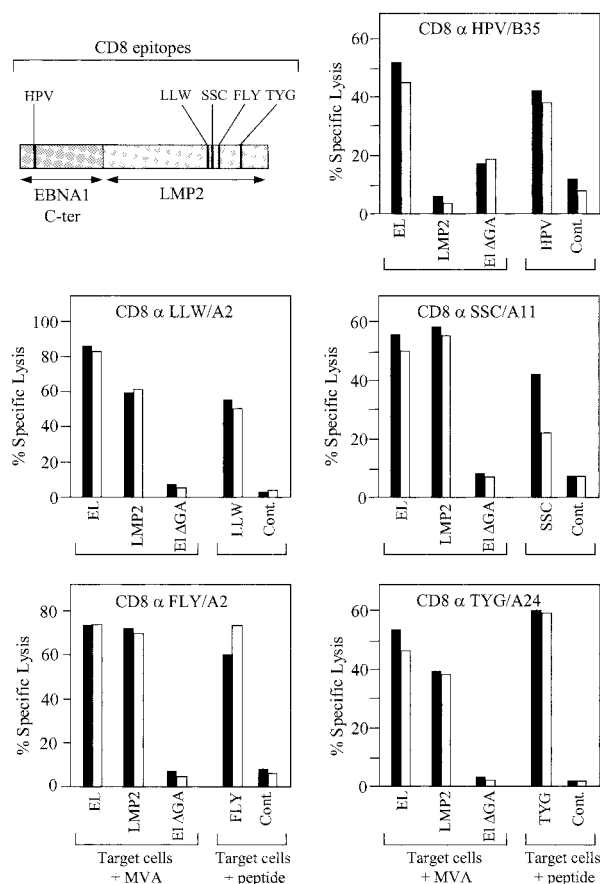


FIG. 3. Processing of endogenously expressed EL via the HLA class I pathway. LCL cells of the appropriate HLA class I type were infected with either MVA-EL, the relevant CD8 epitope peptide, or an equivalent concentration of dimethyl sulfoxide solvent (or irrelevant epitope peptides, data not shown) as a control and then used as targets in 5-h chromium release assays with CD8⁺-T-cell clones against the HPV/B35, LLW/A2, FLY/A2, SSC/A11, and TYG/A24 epitopes. Epitope positions within the EL fusion protein are shown. Results are expressed as percent specific lysis observed at effector-to-target cell ratios of 5:1 (solid bars) and 2.5:1 (open bars).

phorylation, whereas EL and LMP2B were completely inactive.

Presentation of MVA-expressed EL by the HLA class I pathway. We then examined the capacity of EL protein to be processed via the HLA class I pathway when expressed endogenously within target cells from the MVA vector. Figure 3 shows the results of cytotoxicity assays in which MVA-EL-infected LCL cells of the appropriate HLA class I type were used as targets for recognition by CD8⁺-T-cell clones specific for epitope sequences within the EL protein. Four representative CD8 epitopes were tested within the LMP2 sequence: the A2-restricted epitopes LLW (LMP2 amino acids 329 to 337) and FLY (LMP2 amino acids 356 to 364), the A11-restricted epitope SSC (amino acids 340 to 350), and the A24-restricted epitope TYG (LMP2 amino acids 419 to 427). Note that these epitopes are antigenically conserved between Caucasian EBV and Chinese EBV LMP2 genes (21), and so it was possible to assay their presentation with CD8⁺ T-cell clones from Caucasian donors.

In each case, MVA-EL-infected targets were lysed at least as strongly as were targets infected with MVA-LMP2 itself or targets loaded with the epitope peptide; this clearly represents antigen-specific killing because targets infected with MVA-E1ΔGA were not recognized. Note that the EL protein also contains a CD8 epitope within the EBNA1 fragment, the B35.01-restricted epitope HPV (EBNA1 amino acids 407 to 417); although T-cell clones specific for this epitope do not recognize endogenously expressed wild-type EBNA1 because of its protection by the GAR domain, they are known to recognize cells expressing the GAR-deleted form of the protein (2). Here we found that B35.01-positive LCL targets infected with MVA-EL were also recognized by HPV-specific CD8⁺ T cells at least as efficiently or, in the particular assay shown in Fig. 3, more efficiently than MVA-E1ΔGA-infected targets.

For all epitopes tested, therefore, fusion of EBNA1 to LMP2 has not abrogated processing and presentation of the endogenously expressed fusion protein via the HLA class I pathway. Even certain unusual aspects of epitope processing from the wild-type LMP2 protein were maintained. Thus, some of the LMP2 epitopes, for example LLW/A2, are presented via a novel TAP-independent route when processed from their native antigen (20), whereas the HPV/B35 epitope is classically TAP-dependent when processed from EBNA1-ΔGA (2). Assays similar to those shown in Fig. 3 were therefore carried out with the TAP-deficient T2:B35 line as a target. In accord with the idea that TAP dependence or independence is an essential feature of the epitope sequence rather than of the source antigen (20), LLW/A2 was presented from MVA-expressed EL in these TAP-deficient cells, whereas HPV/B35 was not (data not shown).

Subsequent experiments asked whether epitope-specific CD8⁺ memory T cells in the PBMC pool of EBV-immune donors could be reactivated and expanded in vitro by exposure to MVA-EL-infected stimulators. As a source of stimulator cells we used autologous dendritic cell preparations, derived from monocytes by 6 days of culture in granulocyte-macrophage colony-stimulating factor and interleukin-4 and, in view of vaccinia virus' ability to block the dendritic cell maturation response (8), matured in tumor necrosis factor alpha prior to MVA-EL infection. In each case these were compared with unmanipulated autologous LCL cells that are known to be efficient in vitro stimulators of CD8⁺-T-cell memory for EBV latent cycle antigens (37). In vitro responses were measured with the Elispot assay of epitope peptide-induced IFN-γ release because this is more sensitive than cytotoxicity and accurately quantitates the frequency of reactive cells within the culture (50).

Figure 4 shows representative results (expressed as the number of epitope-specific spot-forming cells per 10⁶ total cells in the culture) from assays on day 11 and/or day 17 poststimulation. Donor 1 (HLA-A2 positive, Caucasian) clearly showed more efficient expansion of FLY/A2- and LLW/A2-specific memory cells following stimulation with MVA-EL than with LCL stimulation. Likewise, MVA-EL-infected dendritic cells expanded CD8⁺ memory T cells specific for the SSC/A11 epitope more efficiently than did LCL stimulators in two HLA-A11 individuals, donors 2 and 3 (Caucasian and Chinese, respectively). Such results, seen in several assays of this kind, are particularly significant because these LMP2 epitope reactivi-

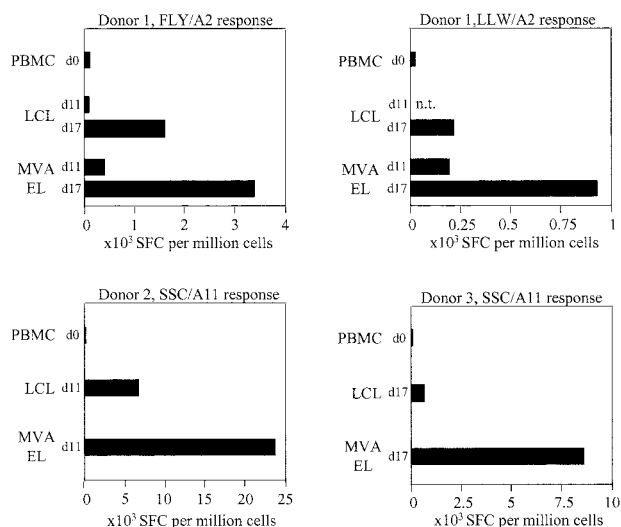


FIG. 4. Immunogenicity of MVA-EL-infected dendritic cells for CD8⁺ memory T cells in vitro. PBMCs from EBV-immune donors of the relevant HLA class I type were cocultured with autologous MVA-EL-infected dendritic cells or with the autologous LCLs, and the frequency of CD8⁺ T cells reactive with relevant CD8 epitopes (FLY/A2, LLW/A2, and SSC/A11) was determined by Elispot assay at the beginning of culture (day 0) and after 11 or 17 days. In all assays, phytohemagglutinin was used as a positive control stimulus, and irrelevant epitope peptides were used as negative controls (data not shown). Results are expressed as number of epitope-specific spot-forming cells (SFC) per million cells in the culture. HLA class I types of donors are: donor 1, A2, A32, B44; donor 2, A1, A11, B7, B35; donor 3, A11 positive, full type not determined.

ties are always subdominant components of EBV-specific memory, with initial levels of reactive cells (25 to 100 spot-forming cells per 10⁶ PBMCs) being much lower than levels typically seen against immunodominant epitopes from the EBNA 3A, 3B, and 3C proteins (50).

In this respect, MVA-EL-infected dendritic cells proved just as efficient stimulators of LMP2-specific CD8⁺ T cells as LMP2 peptide-loaded dendritic cells in earlier work (36), and both were much more efficient than LCL stimulators, where the in vitro response tends to be dominated by EBNA3-specific reactivities. Furthermore, LMP2 epitope-specific CD8⁺ T-cell clones could be established from the MVA-EL-stimulated cultures, and these proved to be functionally similar to LMP2-specific clones, of the kind used in Fig. 3, which had been isolated from LCL-stimulated polyclonal populations (21). In common with CD8⁺ T-cell clones against a number of EBV latent-protein epitopes (14), such LMP2-specific clones do not kill HLA-matched LCLs in conventional 5-h chromium release assays despite the fact that there is some endogenous expression of the appropriate viral target antigen from the resident EBV genome. However, such clones clearly do recognize such LCLs specifically by overnight interferon gamma release and, furthermore, do show specific inhibition of LCL outgrowth in cocultivation assays (S. P. Lee, unpublished data). We therefore infer that such T cells would be effective against EBV-infected target cells in vivo.

Presentation of MVA-expressed EL by the HLA class II pathway. Since previous work has shown EBNA1 to be an

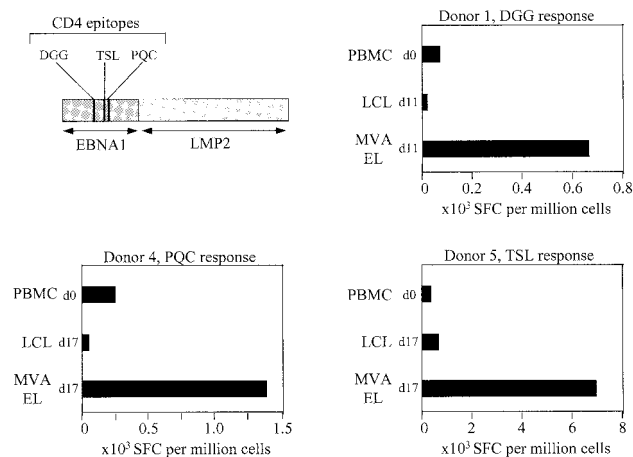


FIG. 5. Immunogenicity of MVA-EL-infected dendritic cells for CD4⁺ memory T cells in vitro. CD8-depleted PBMCs from EBV-immune donors of the relevant HLA class II type were cocultured with autologous MVA-EL-infected dendritic cells or with the autologous LCLs, and responses were determined by Elispot assay as in Fig. 4. Restricting alleles for the EBNA1 epitopes are DGG/not defined, PQC/DR14, and TSL/DR103. HLA class II types of donors are: donor 1, DR7, DR15, DQ2, DQ6; donor 4, DR11, DR14, DQ5, DQ7; donor 5, DR103, DR12, DQ5, DQ7.

important source of CD4⁺-T-cell epitopes in both Caucasian and Chinese individuals (21; X. R. Lin et al., unpublished data), we carried out a parallel series of experiments comparing the ability of MVA-EL-infected dendritic cells and the unmanipulated autologous LCLs to stimulate and expand EBNA1 epitope-specific CD4⁺ T cells from immune donors. The experimental design was essentially similar to that described for the previous set of experiments. Representative results for three EBNA1 CD4 epitopes, all lying within the EL fusion protein sequence, are shown in Fig. 5.

A significant expansion of epitope-specific CD4⁺-T cells above initial levels was obtained within 11 to 17 days in donor 1 for the DGG epitope (EBNA1 amino acids 455 to 469, restriction not defined), in donor 4 for the HLA-DR14-restricted PQC epitope (EBNA1 amino acids 529 to 543), and in donor 5 for the HLA-DR103-restricted TSL epitope (EBNA1 amino acids 515 to 528). Similar results were obtained for other donors reactive to the HLA-DP-restricted NPK (EBNA1 amino acids 475 to 489) and HLA-DR15-restricted MVF (EBNA1 amino acids 563 to 577) epitopes (data not shown). By contrast, throughout these experiments LCL stimulation itself produced no detectable expansion of EBNA1-specific CD4⁺ T-cell memory. This is consistent with the fact that EBV-specific T-cell preparations generated in vitro by LCL stimulation of immune donor PBMCs tend to be dominated by CD8⁺-T-cell reactivities (37, 38).

The efficiency of CD4⁺-T-cell reactivations by MVA-EL-infected stimulators led us to investigate the possibility that endogenously expressed EL protein was directly accessing the HLA class II presentation pathway. For this purpose, we established a number of EBNA1 epitope-specific CD4⁺-T-cell clones by limiting dilutions of responder cell populations from the above MVA-EL stimulation experiments. Figure 6A shows an example of one such clone, PQC c1, specific for the PQC

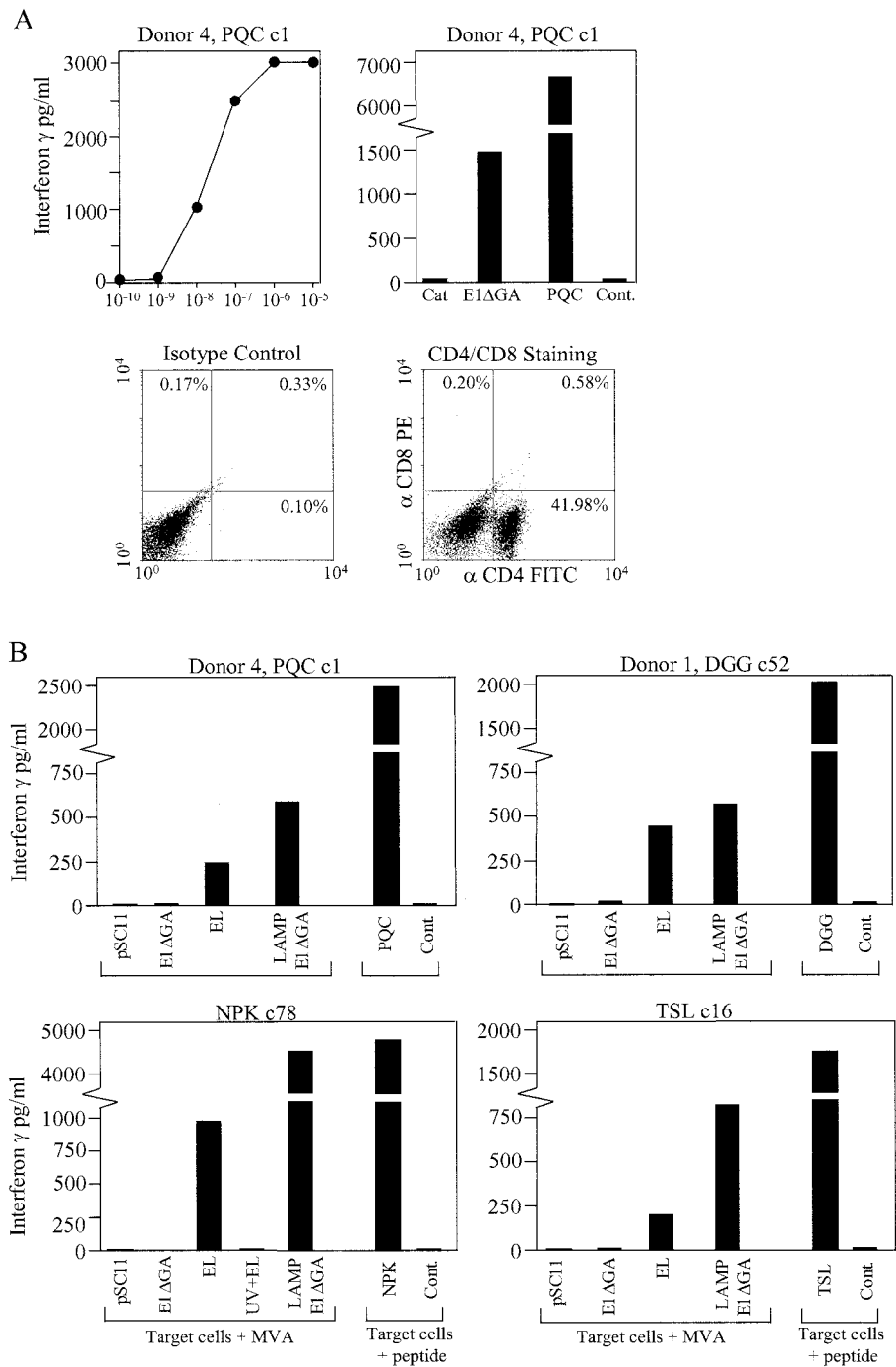


FIG. 6. (A) CD4⁺-T-cell clone derived by MVA-EL-infected dendritic cell stimulation and recognizing the PQC epitope from within EBNA1. The figure shows (i) a titration curve of epitope-specific IFN- γ release after overnight stimulation of the clone by autologous LCL cells preloaded with different concentrations of the PQC peptide (top left), (ii) an assay in which autologous LCL cells were exogenously loaded with baculovirus-expressed EBNA1 Δ GA protein, or with a control protein chloramphenicol acetyltransferase produced similarly in the baculovirus system, or with the PQC peptide, then the cells used as stimulators for the PQC-specific clone, results being expressed as IFN- γ production after overnight incubation (top right), and (iii) fluorescence-activated cell sorting profiles of anti-CD4 and anti-CD8 MAb staining, or staining with appropriate isotype control antibodies; note that the CD4⁺ CD8⁺ signals derive from stimulator LCLs. (B) Processing of endogenously expressed EL via the HLA class II pathway. LCL cells of the appropriate HLA class II type were infected with either MVA-EL, MVA-pSC11, MVA-E1 Δ GA, or MVA-LAMP-E1 Δ GA or loaded with the relevant CD4 epitope peptide or with an equivalent concentration of dimethyl sulfoxide solvent as a control, and then used as stimulators in IFN- γ release assays with CD4⁺-T-cell clones against the EBNA1-derived CD4 epitopes PQC, DGG, NPK, or TSL. Restricting alleles for the DGG, PQC, and TSL epitopes are given in the Fig. 5 legend; the NPK epitope is HLA-DP restricted. The experiment with the NPK clone includes LCLs exposed to UV-irradiated MVA-EL as a further control. Results are expressed as IFN- γ release after 12 h of incubation.

epitope and derived from donor 4. This clone, which fluorescence-activated cell sorting staining confirmed as CD4⁺ CD8⁻, recognized the PQC peptide at concentrations down to 10⁻⁸ M in enzyme-linked immunosorbent assays quantitating IFN- γ release. The clone was confirmed to be antigen specific since it also recognized LCL cells preexposed to an exogenous source of EBNA1 protein (in this case GAR deleted) but not to a control protein.

Figure 6B shows the results obtained in T-cell recognition assays on MVA-EL-infected target cells with CD4⁺-T-cell clones PQC c1 and DGG c52, both generated by MVA-EL stimulation, and other CD4⁺-T-cell clones specific for EBNA1 epitopes, TSL c16 and NPK c78, both generated by direct stimulation of immune donor PBMCs with epitope peptide. Control targets in these experiments were infected with MVA-pSC11 (empty vector), with MVA-E1 Δ GA, and with MVA-LAMP-E1 Δ GA, a virus expressing a form of E1 Δ GA that has been targeted to the MHC class II pathway by fusion with a LAMP sequence. Importantly, all four EBNA1-specific clones showed significant recognition of MVA-EL-infected targets at levels ranging from 20 to 80% of those seen against target cells expressing the MHC class II pathway-directed LAMP-E1 Δ GA protein. Moreover, as illustrated by the data from NPK c78, prior UV inactivation of the virus abolished this recognition of MVA-EL-infected targets, indicating that it was the endogenously expressed EL protein that was being processed for HLA class II presentation and not exogenous protein that could theoretically have been present in the input virus preparation. In the same assays, targets infected with the MVA-E1 Δ GA virus were never recognized, despite the fact that levels of antigen expression were as high as for the MVA-EL virus (see, for example, Fig. 1B).

Intracellular localization of MVA-expressed EL. These findings suggest that fusion to LMP2 had provided the EBNA1 sequence within EL with a direct route into the HLA class II processing pathway, a route which was apparently not accessible to the EBNA1 protein itself. In a final series of experiments we examined the intracellular localization of EL and, for comparison, of E1 Δ GA, LAMP-E1 Δ GA, and LMP2 expressed from MVA vectors in matured dendritic cells and visualized by MAb staining. As illustrated in Fig. 7A, EBNA1-specific MAb staining indicated that EL was concentrated in dense cytoplasmic patches on a background of weak general cytoplasmic fluorescence. This was quite distinct from the exclusively nuclear staining of E1 Δ GA shown by the same MAb and from the weak nuclear plus fine punctate cytoplasmic staining of LAMP-E1 Δ GA. Staining of EL-expressing cells by the LMP2-specific MAb again was concentrated in dense cytoplasmic patches; this was also distinct from the punctate, largely perinuclear staining of wild-type LMP2.

In subsequent experiments (Fig. 7B), EL-expressing cells were stained for the fusion protein with either EBNA1-specific or LMP2-specific MAbs (green signals) and with MAbs to cellular markers of the HLA class II processing pathway (red signals). While staining for HLA class II molecules themselves was predominantly at the cell surface (data not shown), endosomal markers such as EEA1 and CD63 or lysosomal markers such as LAMP1 showed typical distribution within cytoplasmic vesicles. However, none of these markers showed significant colocation with the dense patches of EL staining.

DISCUSSION

One aim of a nasopharyngeal carcinoma therapeutic vaccine will be to elicit or boost CD8⁺-T-cell responses to LMP2. However the important role which CD4⁺ T cells play in the development and maintenance of CD8⁺-T-cell memory (15, 42, 45) emphasizes the likely need for a source of CD4⁺ T-cell epitopes in such a vaccine construct. LMP2 does not appear particularly immunogenic to CD4⁺ T cells. By contrast, EBNA1, an antigen coexpressed with LMP2 in nasopharyngeal carcinoma cells, is a strong CD4⁺ immunogen, with most if not all epitopes located in the C-terminal half of the molecule (21, X. R. Lin, unpublished data). The EL fusion protein described here includes the relevant EBNA1 fragments linked to full-length LMP2.

At the outset we were concerned to minimize the possibility that EL would retain any of the biological functions of its component proteins. The DNA replication, genome segregation, and transcriptional activation functions of EBNA1 require the essential DNA-binding and dimerization domain residues 451 to 641 but are also dependent upon the presence of a Gly-Arg-rich region, residues 325 to 376 and (to differing extents) on the N-terminal residues 8 to 67 (52). We indeed confirmed that EL, which lacks most of the Gly-Arg-rich region and all of the N-terminal domain, had lost transcriptional activating function although, like other C-terminal domain constructs designed as dominant-negative inhibitors of EBNA1 (19), it appears to retain sequence-specific DNA binding activity, here reflected in suppression of an EBV *Bam*HI-Q reporter construct (Fig. 2A). This residual activity is very unlikely to have biologic effects within cells, especially since even wild-type EBNA1 appears not to act directly upon cellular gene transcription (17) and indeed is dispensable for EBV-induced B-cell growth and transformation if its genome maintenance function is rendered redundant by integration of the viral genome into cellular DNA (15). The LMP2 protein also is not required for B-cell transformation (44) but nevertheless does have important signaling functions that are mediated via tyrosine residues 74 and 112 acting as recruitment sites for the Syk and Lyn tyrosine kinases, respectively (25). We confirmed that mutation of both these residues in the EL construct indeed abrogated the signaling capacity of the fusion protein.

The selection of MVA as a vector for EL expression reflects the proven safety and efficacy both of the MVA strain itself as a smallpox vaccine in humans and of MVA recombinants when tested in animal models as prophylactic vaccines for viral and parasitic infections (12, 14, 48, 53) and even as therapeutic vaccines against tumors expressing strong indicator target antigens (3). The present work has focused on in vitro studies with the MVA-EL construct because our priority was to examine the ability of the EL fusion protein to be processed and its constituent epitopes presented to T cells.

We first examined processing via the HLA class I pathway and showed that target cells endogenously expressing EL from the MVA vector were efficiently recognized and killed by CD8⁺-T-cell clones whether specific for the HPV/B35 epitope within the EBNA1 fragment of the fusion protein or specific for any of four LMP2 epitopes tested (Fig. 3). Importantly those included the SSC/A11 and TYG/A24 epitopes that are of particular relevance for a nasopharyngeal carcinoma vaccine

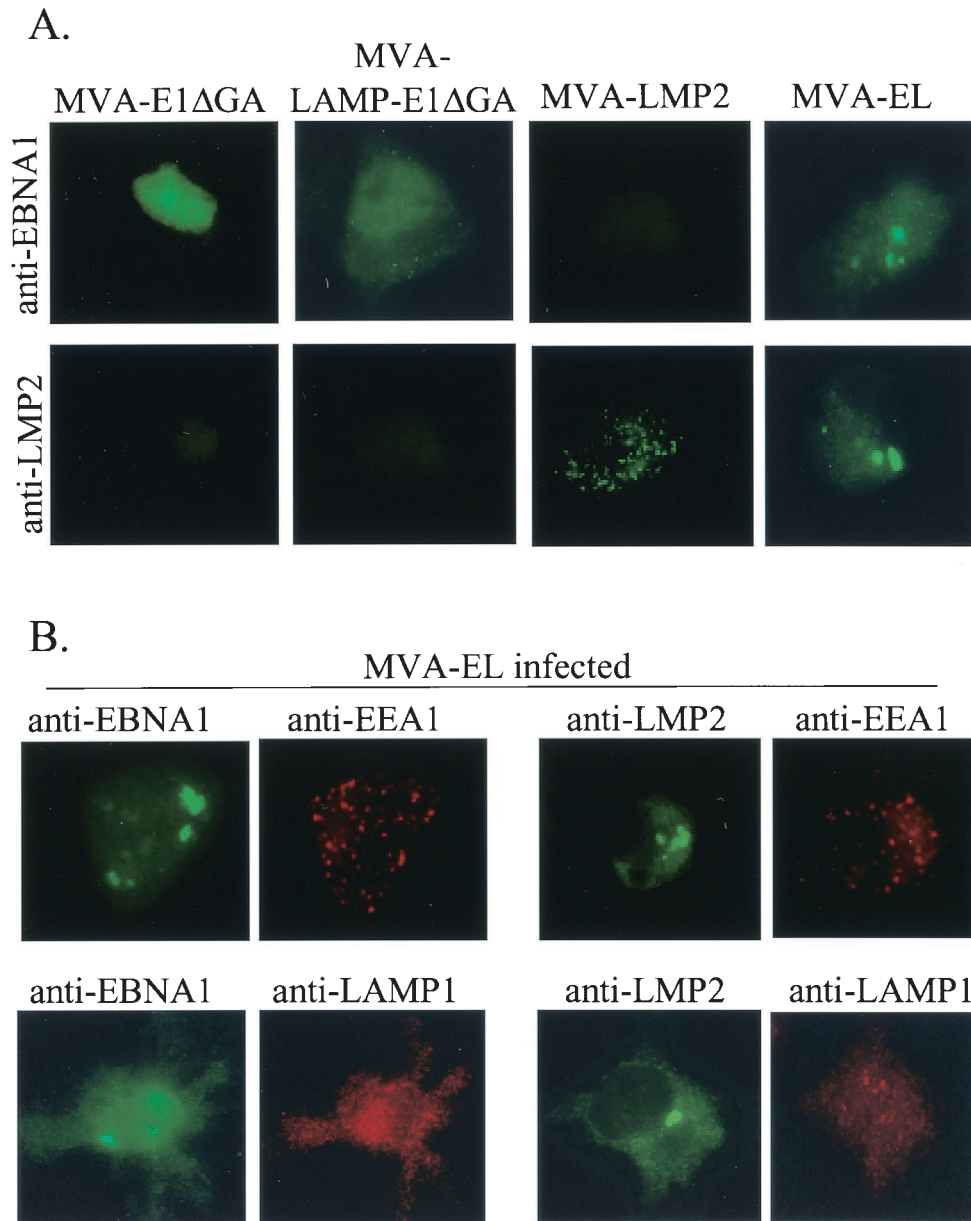


FIG. 7. (A) Immunofluorescence photomicrographs of matured dendritic cells infected with MVA-EL, MVA-E1ΔGA, MVA-LAMP-E1ΔGA, or MVA-LMP2 and then stained either with the EBNA1-specific MAb 1H4-1 or with the LMP2-specific MAb 14B7. (B) Two-color immunofluorescence photomicrographs of matured dendritic cells infected with MVA-EL and then costained for either EBNA1 or LMP2 as above (green fluorescence) and with the endosomal/lysosomal marker EEA1 or LAMP1 (red fluorescence).

because of the high frequency of the HLA-A11 and A24 alleles in the Chinese population (21). Subsequent experiments showed that endogenously expressed EL was also capable of reactivating LMP2 epitope-specific CD8⁺ T-cell memory responses in vitro, in this case with autologous dendritic cells as the MVA-EL-infected stimulator population and comparing this with the unmanipulated LCL. Just as in earlier work with dendritic cells loaded with LMP2 epitope peptides as stimulators (36), dendritic cells expressing EL were more efficient than LCL cells at reactivating the low numbers of LMP2 epitope-specific memory T cells typically found in the blood of healthy virus carriers (Fig. 4). Furthermore, these reactivated effectors

were functionally equivalent to LMP2 epitope-specific CD8⁺ T cells isolated by cloning from LCL-stimulated polyclonal populations (21).

Parallel in vitro stimulation experiments designed to study EL presentation via the HLA class II pathway were particularly interesting. They showed that MVA-EL-infected dendritic cells induced very efficient reactivation of CD4⁺ memory T cells for all five EBNA1 epitopes tested, whereas there was little or no evidence of such reactivation induced by the LCL within the same time scale (Fig. 5). It is worth noting that, although these five epitopes were originally identified from work on Caucasian donors, three of them (PQC, TSL and

NPK) are also among the range of EBNA1 epitopes commonly seen by the CD4⁺ T-cell response in Chinese individuals (X. R. Lin et al., unpublished data). Subsequent experiments strongly suggested that the endogenously expressed EL protein was directly accessing the HLA class II pathway. Thus, EBNA1 epitope-specific CD4⁺ T-cell clones showed significant recognition of MVA-EL-infected targets within 12 h of infection, and this clearly was dependent upon de novo EL expression (Fig. 6).

It is formally possible that, even within this short time, recognition stems from the release of EL protein from infected cells and its uptake and reprocessing as exogenous antigen by neighboring cells. However this seems very unlikely given the fact that cells infected in parallel with MVA expressing equivalent levels of a GAR-deleted EBNA1 protein (which would be similarly available for release and reprocessing) were never recognized. Note that the antigen-presenting cells in this experiment were the autologous EBV-transformed LCLs, and these were also not detectably recognized by the CD4⁺-T-cell clones despite endogenous expression of native EBNA1 from the resident EBV genome. While particular high-affinity CD4⁺-T-cell clones against EBNA1 epitopes have been reported to show LCL recognition (31, 34), this is not true of most EBNA1-specific clones in our experience. In any case, the present data clearly show that incorporation into the EL fusion protein has given EBNA1 epitopes much improved access to the HLA class II presentation pathway, mediating levels of CD4⁺-T-cell recognition that were 20 to 80% of that seen when the GAR-deleted EBNA1 protein was deliberately targeted to the class II pathway by fusion with a LAMP sequence.

Interestingly, fluorescence studies showed that fusion to LMP2 had dramatically altered the location of EBNA1 staining from exclusively nuclear to dense cytoplasmic patches on a background of weak cytoplasmic fluorescence. However, this did not simply reproduce the pattern of wild-type LMP2 staining, which, as others have reported (6, 26), is found in abundant intracellular vesicles at least partly colocalized with cellular endosomal proteins such as EEA1 and CD63. Nor did it follow the pattern of LAMP-targeted EBNA1, which showed more discrete cytoplasmic vesicular staining consistent with endosome/lysosome targeting. The precise nature of the dense patches of EL staining, apparent after MVA-EL infection of both dendritic cells (Fig. 7) and A431 epithelial cells (data not shown) remains to be determined.

It is nevertheless clear that the fusion protein can access both the HLA class I and class II processing pathways in antigen-presenting cells and that MVA-EL-infected dendritic cells can efficiently reactivate CD4⁺ memory T cells specific for EBNA1 epitopes and CD8⁺ memory T cells specific for LMP2 epitopes. Such a virus has potential as a therapeutic vaccine against the EBV-associated tumor nasopharyngeal carcinoma, either to boost the relevant components of immune T-cell memory in nasopharyngeal carcinoma patients or, perhaps as part of a heterologous prime-boost strategy (28, 41), to elicit and sustain new responses capable of targeting tumor cells.

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